

J of Immunology 108:253-260, 1972

AFRRI SR71-7
MAY 1971

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**ALTERATION IN GRAFT VERSUS HOST
REACTIVITY AND IN HEMATOPOIETIC
STEM CELLS OF SPLEEN CELL INOCULA
FROM DONOR MICE PRETREATED
WITH PERTUSSIS ANTIGEN**

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Atomic Support Agency
Bethesda, Maryland

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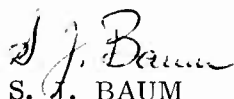
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May 1971

ALTERATION IN GRAFT VERSUS HOST REACTIVITY AND IN
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FROM DONOR MICE PRETREATED WITH
PERTUSSIS ANTIGEN

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ACKNOWLEDGMENT

The interest and encouragement of S. J. Baum, the technical assistance of R. T. Brandenburg, the statistical analyses performed by S. G. Levin and the editorial assistance of C. H. Poppe are gratefully acknowledged.

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FOREWORD

(Nontechnical summary)

The transplantation of bone marrow from another human is the only specific treatment available to personnel who have received radiation sufficient to cause death from the failure of the blood cell forming (hematopoietic) function of the bone marrow. The transplanted donor cells will generally proliferate in such a host when the host's own marrow is unable to function; a portion of the donor cells, the immunocompetent cells, however, may recognize the host as foreign tissue and mount an immune attack. The outcome is usually fatal. The attack is known as the graft versus host reaction (GVHR); it is analogous to the more familiar host versus graft reaction in which host immunocompetent cells recognize and reject as foreign a transplanted organ. The GVHR may sometimes be reduced if the host and donor tissue types can be matched. Neither this laborious procedure nor other available treatments appear likely to eliminate by themselves the problem of GVHR. We therefore sought a way to provide tissue for transplantation with adequate hematopoietic stem cells but with reduced GVHR. Our studies were designed to test the hypothesis that a dose of pertussis (whooping cough) vaccine administered to donor animals would cause large numbers of immunocompetent lymphocytes to move out of the hematopoietic tissue and into the blood stream. This might reduce the number of GVHR cells in hematopoietic transplants but retain or increase the number of functional hematopoietic stem cells.

Donor mice were treated with either a saline (control) solution or a pertussis vaccine preparation. Donor spleen cells, which in the mouse resemble human bone

marrow in their functions, were then transplanted by injection into recipient mice in experiments designed to test GVHR and hematopoietic stem cells quantitatively and specifically.

We observed that pertussis vaccination reduced the proportion of GVHR cells and increased the proportion of hematopoietic stem cells in donor mouse spleens. These two effects improved the treatment value of this hematopoietic transplant thirteenfold compared with control transplants. The findings suggest a general approach to GVHR which may have implications for the use of hematopoietic transplantation in cancer therapy, some anemias, and other conditions in addition to radiation injury. More animal experiments will be necessary to clarify the mechanism of the effects observed and to evaluate the clinical implications these results may have.

ABSTRACT

The graft versus host reactivity (GVHR) and hematopoietic stem cells of spleen cell inocula were assayed 3 days after pretreatment of donor mice with heated or unheated pertussis vaccine. Unheated vaccine reduced GVHR to 17 percent of control reactivity, measured by the Simonsen spleen weight assay, and increased 2.2-fold the proportion of hematopoietic stem cells, assayed as colony forming units (CFU). Heated vaccine, which did not produce the lymphocytosis induced in donors by unheated vaccine, reduced GVHR to 38 percent of control reactivity, and increased the proportion of CFU 3.8-fold. Alternative mechanisms for these effects are discussed.

I. INTRODUCTION

Allogeneic bone marrow transplants prolong survival in rodents with radiation-induced hematopoietic aplasia^{20,26} through establishment of hematopoietic chimerism by the transplant.^{2,28} However, in humans with hematopoietic aplasia, clinical trials of bone marrow transplantation demonstrated that the establishment of a histoincompatible marrow allograft leads to an acute fatal secondary syndrome.^{30,31,51} The syndrome is attributed to an immunologic graft versus host reaction (GVHR).^{42,45,52} Recently introduced histocompatibility matching methods offer hope of alleviating the syndrome when suitable donors are available.^{1,8,15,18} Additional methods of reducing the GVHR are required especially for the majority of recipients for whom matched donors may not be available. Several methods of reducing the GVHR are under investigation in other laboratories and have been recently summarized.¹⁰

Pertussis vaccine mobilizes mouse lymphocytes³⁸ of a thymus-dependent population²¹ from the hematopoietic organs into the blood stream. Thymus-dependent lymphocytes may initiate cellular immune reactions^{14,17,37} of which the GVHR is an example.⁵⁰ We therefore hypothesized that pertussis vaccine pretreatment of donor animals would deplete donor organs of GVHR cells while retaining significant hematopoietic stem cells. Transplantation of mouse spleen cells from pretreated donors was chosen as an experimental model because mouse spleen resembles human bone marrow in containing both hematopoietic stem cells and cells causing acute GVHR.⁴⁰ The studies reported in this communication showed that pertussis vaccination of donor mice leads to a reduction of GVHR and to an increase in hematopoietic stem cells of donor mouse spleen. It was also shown that lymphocyte mobilization could only partially account for these effects.

II. MATERIALS AND METHODS

Animals. C57BL/6J ♀ (H-2^b), DBA/2J ♂ (H-2^d), and the F₁ ♀ hybrid offspring of these strains of mice (designated B6D2F₁/J) were purchased from the Jackson Laboratory, Bar Harbor, Maine. The animals were allowed food,* and water adjusted to pH 3 with hydrochloric acid, ad libitum. Donors and irradiated recipients were used at 9-12 weeks of age, weighing 17-24 g. Neonatal litters of B6D2F₁ mice were bred in our laboratory from Jackson stock; they were used in assays for GVHR at 6-10 days of age and were nursed by the mother until completion of the assays.

Radiation. Irradiated animals received 850 rads (LD_{95-100/30}) produced by a General Electric Maxitron x-ray machine with the following characteristics: 250 kVp, 30 mA, 1.2 mm Be + 0.95 mm Cu filtration (HVL = 1.91 mm Cu), target to subject midline distance 67.5 cm. The midline absorbed dose rate was 56.2 rads/minute.

Bordetella pertussis pretreatment of donor mice. B. pertussis vaccine† containing 200×10^9 killed organisms/ml (6 mg protein/ml)‡ in 0.01 percent thimerosal was diluted to 5 volumes with 0.9 percent sodium chloride just prior to use. Heated pertussis vaccine was prepared by heating diluted vaccine at 100°C for 30 minutes. Heating in this manner removes the capacity of the vaccine to mobilize lymphocytes;³⁸ it also removes immunogenicity for specific protection from pertussis infection, and a number of other activities of the vaccine.²³ Ten billion organisms contained in 0.25 ml of the unheated or heated diluted vaccine were injected into a tail vein of

* D & G Rat and Mouse Diet, specific pathogen free. G. L. Baking Company, Frederick, Maryland

† Lot No. 985739, kindly supplied by Dr. H. B. Devlin, Parke, Davis and Company, Detroit, Michigan

‡ Kindly determined by Dr. W. D. Skidmore by the method of Lowry et al.²⁷

experimental donors; control donors received 0.25 ml saline. Three days after injection, the animals were sacrificed by cervical dislocation and the spleens were removed and weighed. The number of tail vein blood leukocytes of about one-half the donor animals were counted as an index of stability of the vaccine used. The potency of the vaccine for producing leukocytosis or increased spleen weights was stable during the course of the studies.

Cell suspensions. The spleens removed from donor animals were weighed and maintained subsequently in Hanks' balanced salt solution (HBSS) in an ice bath. The spleens were minced with scissors and expressed through 400-mesh nylon gauze. The resulting suspension of individual cells was washed by dilution in 30 volumes of HBSS followed by centrifugation at 330 g for 8 minutes at 4°C. The supernatant was then decanted; this procedure was repeated for a total of three washes. The cells were then resuspended with HBSS to about 0.3 ml/spleen. An aliquot was diluted in a red cell pipet with 0.4 percent trypan blue in saline. Nucleated cells excluding trypan blue were counted in a hemacytometer 1-3 minutes after dilution. The suspensions were then diluted with HBSS to contain the desired number of nucleated cells either in 0.4 ml for tail vein injection into adult irradiated recipients or in 0.1 ml for intra-peritoneal injection into neonates.

Assay of GVHR. A modification of the spleen weight assay of Simonsen^{34, 46} was performed. The assay is based on the early finding of splenomegaly in neonatal mice following injection of histoincompatible adult lymphoid cells. Doses of 2, 5, 10 or 20 million spleen cells from experimental or control donors were injected intra-peritoneally into 6- to 10-day-old B6D2F₁ mice. Three or four littermates were left

uninjected. Litters of fewer than six animals were not used and animals over 10 in an individual litter were discarded. After the injection, the litter was returned to the mother for 9 days. The litter was then sacrificed and individual spleen and body weights were determined. The spleen index for each of the injected animals was calculated by dividing its spleen to body weight ratio by the average of the spleen to body weight ratios of the uninjected littermates.

Assays of hematopoietic stem cells. The hematopoietic stem cells of experimental or control donor spleen cell suspensions were determined by intravenous injections of 2.0×10^5 or 1.0×10^6 cells into lethally irradiated (850 rads) syngeneic recipients. Irradiated control recipients received 0.4 ml HBSS. Untreated unirradiated control mice were also maintained and subjected to the hematopoietic assays. On the 10th day, 1 μ Ci of $^{59}\text{Fe}^*$ was injected subcutaneously.²⁹ Six hours later the mice were sacrificed and spleen and body weights determined. The radioactivity of a humerus and the spleen was determined in a scintillation counter. The spleens were fixed in Bouin's solution and the number of macroscopic surface nodules on the spleen, each representing a hematopoietic colony forming unit (CFU), were counted.⁴⁹

In preliminary studies, the hematopoietic assays of C57BL/6J mice used as donors in the GVHR assay were compared with those of B6D2F₁/J mice. Since spleen cells of pertussis vaccine pretreated or control donors from both strains produced similar hematopoietic responses in syngeneic recipients, all subsequent studies reported here were performed with B6D2F₁/J donors in order to avoid the high early postirradiation mortality of C57BL/6 mice.³²

* As ferrous citrate at pH 6.8. New England Nuclear Corporation, Boston, Massachusetts

Cytological studies. In separate experiments, animals of each of the donor strains were injected with either unheated pertussis vaccine, heated pertussis vaccine or saline as described above. At 3 days, total and differential leukocyte counts were determined, body and spleen weights were measured, and a touch preparation of the spleen was stained with Wright-Leishman's stain. Total nucleated cells per spleen were calculated from counts of aliquots of pooled spleen suspensions diluted in 3 per-cent acetic acid.

Statistical analyses. The t-test was used to compare averages of values obtained. Differences are reported as significant when $p < 0.05$.

III. RESULTS

Effects of the vaccine preparations used on donor mice are shown in Figure 1. Both the heated and unheated pertussis vaccine caused a significant increase over values from control animals in average spleen weight and in the number of nucleated cells per spleen. Body weights were not significantly affected. Examination of the tail vein blood showed approximately a ninefold increase in peripheral leukocytes, with an eightfold increase in the sum of lymphocytes and monocytes in the animals treated with unheated vaccine. The heated vaccine did not significantly change the blood counts.

Cytological examination of touch preparations of the spleen showed increases over controls in immature cells of the myelocytic and lymphocytic series and in the numbers of mature neutrophils in the spleens of the animals treated with heated or unheated vaccine.

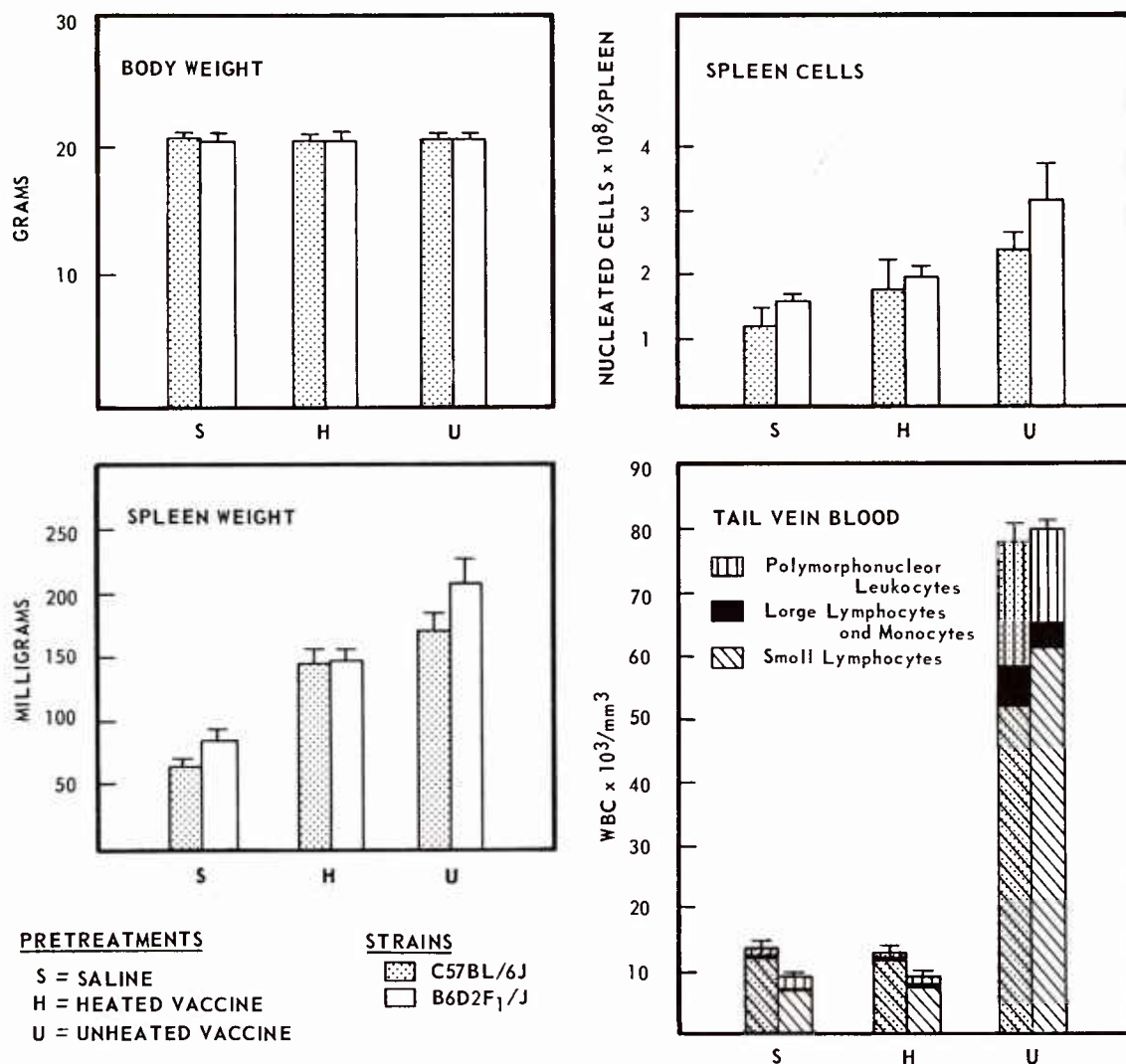


Figure 1. Responses of donor mice to pretreatments. Average values for four or five recipients are shown 3 days after injection of heated or unheated pertussis vaccine or saline solution. The vertical bar (\top) denotes 1 S.E. except in the case of spleen cells where 1 S.D. for four replicate measurements from pooled spleens is shown.

Results of the assay of the GVHR are shown in Figure 2 for spleen cells of C57BL/6J donors from each of the pretreatment groups injected into B6D2F₁ recipients. Compared with the control curve, both of the experimental dose response curves indicate that more experimental cells are required to produce a given spleen

index. Statistical comparison of the slopes of the three regression lines¹⁶ shows that they are not significantly different. The relative reactivities of the various cell populations may therefore be expressed as the ratio of the numbers of control cells to experimental cells required to produce any spleen index within the range of indices associated with parallel dose response curves. The ratio may be obtained through an equation derived for calculation of the distance between regression lines.¹⁶ Substituting the GVHR data shown in Figure 2 into the equation, one obtains the ratios of 0.38 for the numbers of control cells to cells from donors pretreated with heated vaccine, and 0.17 for the numbers of control cells to cells from donors pretreated with unheated vaccine.

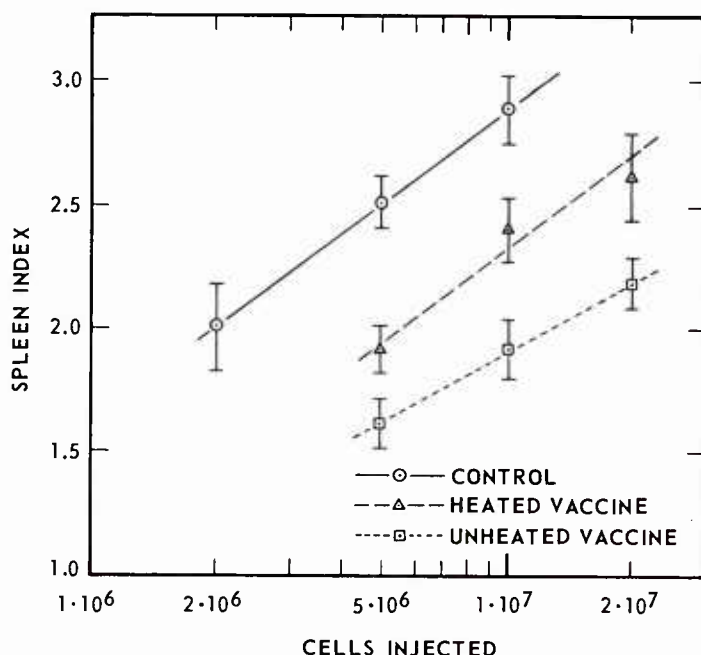


Figure 2. GVHR of C57BL/6J donor spleen cells assayed in B6D2F₁ neonatal recipients. Cells from control donors pretreated with saline or from experimental donors pretreated with either heated or unheated pertussis vaccine were injected into B6D2F₁ neonates in graduated doses in six experiments. Each point represents the average spleen index \pm 1 S. E. of a total of 30-40 recipients. Regression lines were fitted by the method of least squares.

As a control experiment for the assay of the GVHR, separate assays reported in Table I were performed in B6D2F₁ neonates but, instead of the parental strain, donors were syngeneic B6D2F₁/J adults receiving pretreatment identical to that of the

C57BL/6J parental strain donors. The observed absence of significant changes in the average spleen indices of neonatal B6D2F₁ recipients after injection with syngeneic cells from control or experimental donors is consistent with the interpretation that the splenomegaly induced in the spleen assay of the GVHR required a histocompatibility difference between donor and recipient, and excludes the possibility that the GVHR assay shown in Figure 2 was influenced by factitious splenomegaly such as might be caused by vaccine carried with the donor cells.

Table I. GVHR of B6D2F₁/J Donor Spleen Cells Assayed in Syngeneic Recipients

Donor pretreatment (I. V.)	Recipient cell dose (millions)	Number of recipients	Average spleen index \pm 1 S. E.
Saline	2	6	$1.00 \pm 0.07^*$
	5	5	1.14 ± 0.08
	10	5	1.00 ± 0.09
Heated pertussis vaccine	5	5	0.99 ± 0.10
	10	6	0.99 ± 0.02
	20	6	1.08 ± 0.05
Unheated pertussis vaccine	5	5	0.97 ± 0.07
	10	6	0.97 ± 0.08
	20	6	1.12 ± 0.08

* None of the averages differ significantly from 1.0

Hematopoietic stem cells of B6D2F₁/J spleen cells from control and experimental donors are shown in Figure 3. Although the standard errors of the average indices in these experiments were relatively large, the spleen colony counts, splenic iron uptake and spleen weight assays (Figure 3A-C) were in agreement when experimental inocula were compared with control cell inocula. Inocula from donors pretreated with heated

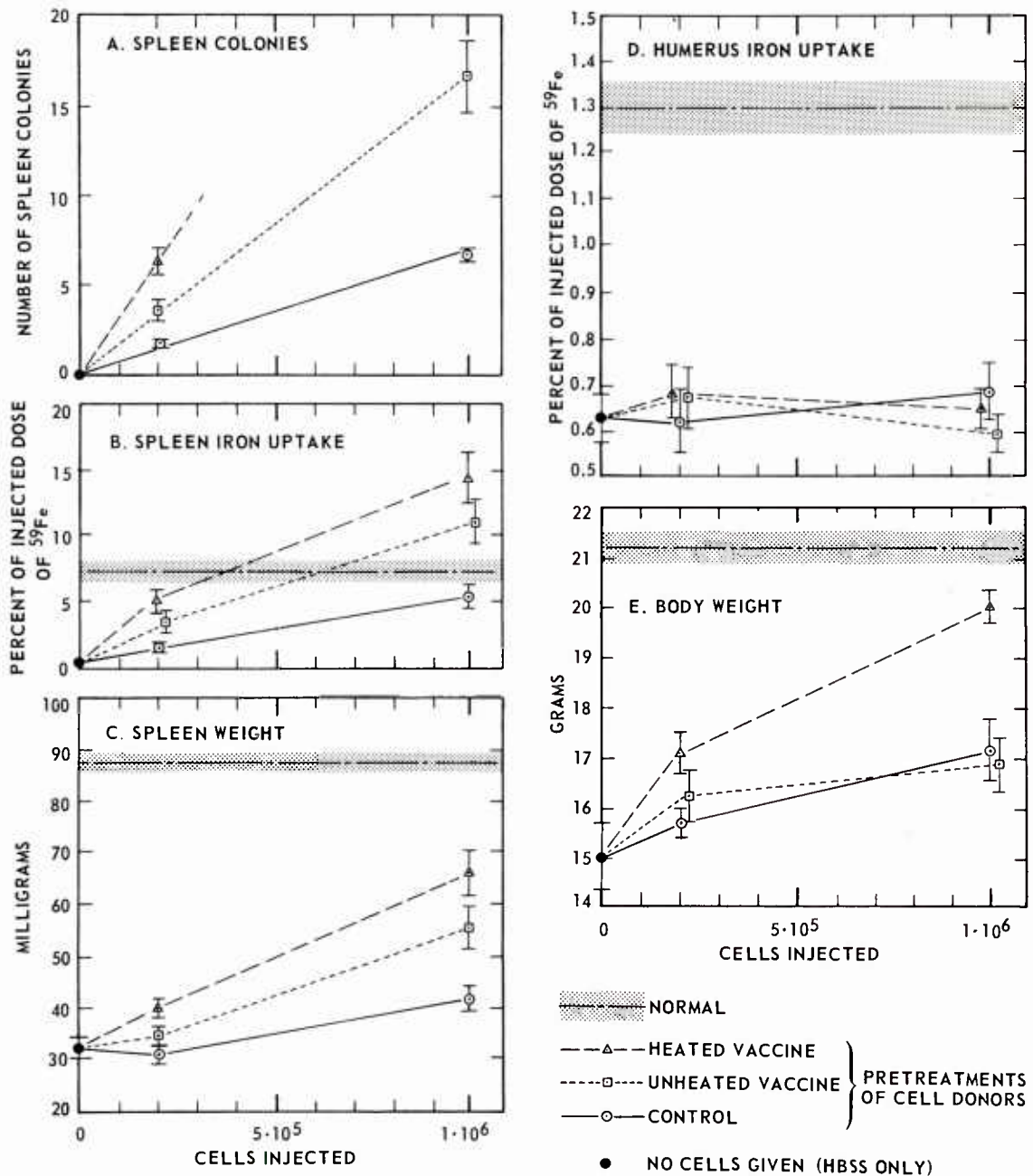


Figure 3. Indices of hematopoietic stem cells of B6D2F₁/J donor spleen cells assayed in syngeneic recipients. Recipients were lethally irradiated, then injected with HBSS or with spleen cell suspensions from control donors pretreated with saline or from experimental donors pretreated with either heated or unheated pertussis vaccine. From 19-23 survivors in each group were assayed on the 10th day for (A) spleen colonies, (B) spleen ⁵⁹Fe uptake, (C) spleen weight, (D) humerus ⁵⁹Fe uptake, and (E) body weight.

vaccine produced the largest average indices, followed by inocula from donors pretreated with unheated vaccine. Control cell inocula produced the smallest indices. Inocula from donors pretreated with heated vaccine resulted in increased average body weights in the recipients but inocula from donors pretreated with unheated vaccine resulted in average body weights about the same as those resulting from control cell inocula.

Since the number of CFU counted is linearly related to the number of hematopoietic stem cells injected⁴⁹ up to a saturation point of about 25-30 CFU per spleen, quantitative comparisons of the stem cell content of the various inocula may be drawn. Thus, when effects at a dose of 0.2 million cells are compared, spleen cells from donors pretreated with unheated or heated pertussis vaccine produce respectively 2.2 or 3.8 times as many CFU as the cells from control donors (Figure 3A).

The determinations of splenic iron uptake were controlled by assessment of marrow iron uptake (Figure 3D). Marrow erythropoiesis, as reflected in the uptake by the humerus, remained at the reduced level of the irradiated mice which received HBSS without cells. The lack of evidence of change in marrow iron uptake under any of the experimental conditions employed excludes the possibility that differences in recipient spleen erythropoiesis observed were merely reflecting proportionate changes in marrow erythropoiesis without actual changes in the total effective erythropoietic stem cells transplanted.

The combined effects of the vaccine pretreatments on donor GVHR and hematopoietic stem cells may be summarized in the manner of Dicke et al.⁹ as the CFU to GVHR ratio. The ratios shown in Table II represent an approximate index of the

therapeutic efficacy of the donor spleen cells in hematopoietic transplantation. A ratio of 13 was found for unheated pertussis vaccine pretreatment and 10 for heated pertussis vaccine pretreatment, compared with the control index of 1.

Donor pretreatment (I. V.)	Fraction of control index		Ratio* CFU to GVHR
	CFU	GVHR	
Heated pertussis vaccine	3.8	0.38	10
Unheated pertussis vaccine	2.2	0.17	13

Table II. Summary of Effects of Donor Pretreatments on GVHR and Hematopoietic Stem Cells in Spleen Cell Inocula

* Ratio is 1 for control inoculum

IV. DISCUSSION

The present studies showed that following pertussis vaccination, the GVHR of donor mouse spleen cells was reduced to 17 percent of control reactivity when the cells were transplanted across an H-2 histocompatibility difference. Pertussis vaccination was also shown to lead to more than twice the number of hematopoietic stem cells in donor spleens when assayed as splenic CFU in irradiated syngeneic recipients. Each of these findings will be discussed separately, followed by a discussion of their implications when considered together.

Reduction in GVHR. Other evidence had suggested that the increase in blood lymphocyte counts following pertussis vaccination results through mobilization of thymus-dependent lymphocytes,^{21, 38} the cell population thought to cause the GVHR.^{14, 17, 37} The vaccine used in the present studies increased donor blood lymphocyte counts, and also reduced the proportion of GVHR cells in donor spleens. When the vaccine was heated to remove its capacity to mobilize lymphocytes, however, it was still effective in reducing the GVHR to 38 percent of control reactivity.

Therefore, not all of the effect of the pertussis vaccine on the GVHR is likely to be due to the mobilization of lymphocytes.

If mobilization of the GVHR cells into the peripheral blood is important in the effect of the unheated vaccine in reducing the GVHR of the donor spleen, one would predict that the net GVHR of the peripheral blood would be increased. Prior comparisons of the proportion of GVHR cells of peripheral blood from pertussis vaccine pretreated and control donors showed decreased reactivity in the mouse⁶ but increased reactivity in the rat.²⁴ To quantitate the total blood GVHR relative to control or other organ reactivity, however, it would be necessary to know the volume of distribution of the reactive cells in the donor and to use a GVHR assay demonstrated to produce effects dependent upon the number of reactive cells injected. In neither of the studies of peripheral blood were these requirements met. Thus, it remains to be answered whether there is an increase in the total number of GVHR cells in the peripheral blood after pertussis vaccination.

Since we have no direct proof that unheated vaccine shifts GVHR cells into the blood, and since the heated vaccine reduced spleen GVHR without apparently mobilizing cells, mechanisms other than lymphocyte mobilization should be considered to account at least partially for the ability of the vaccine to reduce the GVHR. A simple alternative mechanism would be the dilution in the donor spleen of GVHR cells by unreactive cells. This would lead to a reduction in the proportion of GVHR cells.

Unheated vaccine led to a 2.1-fold increase in the number of spleen cells, which could account for a reduction to as little as 48 percent of control reactivity; heated vaccine led to a 1.6-fold increase in the number of spleen cells, which could account

for a reduction to about 63 percent of control reactivity. Thus, dilution alone could account for a major portion of the observed reduction in GVHR if a large portion of the additional cells in the spleens were inactive in the assay.

A third possible mechanism for the observed reduction in the GVHR is reduced entry of the parental spleen GVHR cells into the spleens of the F_1 neonatal recipients. Recipient splenomegaly and thus apparent GVHR would thereby be diminished. This may be unlikely if the behavior of our spleen cells is comparable to that reported by Morse and Barron for labeled lymphocytes from the peripheral blood of animals undergoing pertussis vaccine-induced lymphocytosis. Compared with controls there was no reported difference in the entry of these peripheral blood cells into the spleens of adult syngeneic recipients.³⁹

A fourth possibility is that pertussis vaccine reduces the GVHR by inactivating GVHR cells through antigenic competition with recipient histocompatibility antigens. The reduction of GVHR by a variety of other antigens under a variety of conditions has been attributed to antigenic competition.^{19,25,36} Antigenic competition might also explain the finding that under some conditions guinea pigs receiving pertussis vaccine have shown diminished tuberculin reactivity¹² and the finding that fowl receiving pertussis vaccine showed diminished cutaneous lymphocyte transfer reactions.⁴³ The precise mechanism of antigenic competition in cellular immune responses is, however, not clear.

In contrast to the immunosuppressive effects of pertussis vaccine in reducing the GVHR, the vaccine is active as an adjuvant in humoral immune responses.^{11,41} Under conditions comparable to ours, Cantor et al.⁵ showed pretreatment by the

adjuvant polyinosinic-polycytidylic acid given to donor BALB/c mice increased the GVHR of donor spleen cells. Similarly, an adjuvant effect of the pertussis vaccine in cellular immunity would have been expected to lead to an increase rather than a decrease in the GVHR under the conditions of the present experiments. Either our vaccine was not effective as an adjuvant under the present conditions or a quantitatively greater immunosuppressive effect may have obscured its adjuvanticity.

Increase in indices of hematopoietic stem cells. Pretreatment of donor mice with unheated pertussis vaccine more than doubled the proportion of hematopoietic stem cells in the spleen cell inoculum, but pretreatment with heated vaccine more than tripled this proportion. The difference in the activities of the two preparations could be accounted for if, as in the case of lymphocyte mobilization, the unheated vaccine mobilizes hematopoietic stem cells and the heated vaccine does not. Given equal stimulation of hematopoietic stem cells, the spleens from the animals in which the cells were not mobilized would contain more stem cells. This hypothesis is in accord with prior findings that unheated vaccine leads to an increase in the circulating hematopoietic stem cells of mice.^{3,6} The effect of heated vaccine on circulating stem cells was not reported.

The finding that donor spleen hematopoietic stem cells are increased after pretreatment with either heated or unheated vaccine adds support to the interpretation that endotoxin, which is heat stable,²² may be responsible for the increase in hematopoiesis which pertussis vaccine may produce.¹³ Other bacterial endotoxin preparations^{47,48} as well as a variety of other antigens,^{4,33} stimulate erythropoiesis. Studies using purified B. pertussis endotoxin might clarify this possible relationship.

The observation that recipient bone marrow iron uptake remained depressed to the level of the irradiated controls regardless of the treatment or dose employed is in accord with findings by others that marrow erythropoiesis is not an important component of the erythropoietic recovery of these irradiated mice 10 days after radiation and transplantation of hematopoietic tissue.³⁵

Pertussis vaccination modifying hematopoietic transplants. A theoretical increase in the efficacy of the transplantation of donor spleen cells is suggested by the CFU to GVHR ratio of 13 times that of the control cells. Since reduction in the GVHR may synergistically increase hematopoiesis,^{7, 44} the actual improvement in therapeutic efficacy in allogeneic transplantation may be even greater.

Parallel quantitative measurements of the GVHR and hematopoietic stem cells allow at least approximate comparison of alternative methods of preparing hematopoietic tissue for transplantation. For example, in the studies of Dicke et al.,⁹ mouse spleen cell fractionation on a discontinuous albumin gradient produced a fraction with more than a tenfold increase in hematopoietic cells and less than 10 percent of the GVHR compared to the control suspension. The resulting CFU to GVHR ratio of greater than 100 for the 3-5 percent of cells recovered may be compared with the CFU to GVHR ratio of 13 found in the present study.

The more general use by other investigators of parallel quantitative assays of the GVHR and hematopoiesis would aid in further comparisons of treatments and would facilitate the evaluation of two or more treatment methods applied to the same hematopoietic transplant.

Further studies seem warranted to determine whether pertussis vaccine, its modified fractions, or other agents which duplicate useful properties of the vaccine, may have eventual therapeutic implications in human marrow transplantation.

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DOCUMENT CONTROL DATA - R & D

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1. ORIGINATING ACTIVITY (Corporate author) Armed Forces Radiobiology Research Institute Defense Atomic Support Agency Bethesda, Maryland 20014		2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	
		2b. GROUP N/A	
3. REPORT TITLE ALTERATION IN GRAFT VERSUS HOST REACTIVITY AND IN HEMATOPOIETIC STEM CELLS OF SPLEEN CELL INOCULA FROM DONOR MICE PRETREATED WITH PERTUSSIS ANTIGEN			
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)			
5. AUTHOR(S) (First name, middle initial, last name) E. A. Elkman and R. T. Bowser			
6. REPORT DATE May 1971		7a. TOTAL NO. OF PAGES 30	7b. NO. OF REFS 52
8a. CONTRACT OR GRANT NO.		9a. ORIGINATOR'S REPORT NUMBER(S) AFRRI SR71-7	
b. PROJECT NO. NWER XAXM			
c. Task and Subtask C 903		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
d. Work Unit 01			
10. DISTRIBUTION STATEMENT Approved for public release; distribution unlimited			
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY Director Defense Atomic Support Agency Washington, D. C. 20305	
13. ABSTRACT <p>The graft versus host reactivity (GVHR) and hematopoietic stem cells of spleen cell inocula were assayed 3 days after pretreatment of donor mice with heated or unheated pertussis vaccine. Unheated vaccine reduced GVHR to 17 percent of control reactivity, measured by the Simonsen spleen weight assay, and increased 2.2-fold the proportion of hematopoietic stem cells, assayed as colony forming units (CFU). Heated vaccine, which did not produce the lymphocytosis induced in donors by unheated vaccine, reduced GVHR to 38 percent of control reactivity, and increased the proportion of CFU 3.8-fold. Alternative mechanisms for these effects are discussed.</p>			

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